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Prognostic significance of Hif1α and Glut1 expression in malignant pleural mesothelioma

INAUGURAL-DISSERTATION
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vorgelegt von
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List of abbreviations:

^{18}FDG-PET: ^{18}Fluor-deoxyglucose-positron emission tomography
CEA: carcinoembryonic antigen
EGFR: epidermal growth factor receptor
EMA: epithelial membrane antigen
EPP: extrapleural pleuro-pneumonectomy
Glut1: glucose transporter 1
HGFR: hepatocyte growth factor receptor
Hif1α: hypoxia inducible factor 1α
Hif2α: hypoxia inducible factor 2α
IMIG: International Mesothelioma Interest Group
LDH: lactate dehydrogenase
MAP-kinase: mitogen-activated protein kinase
MPM: malignant pleural mesothelioma
mTOR: mammalian target of Rapamycin
NF2: neurofibromin 2
PDK: pyruvate dehydrogenase kinase
PHD: prolyl hydroxylases
PI3K: phosphoinositide-3-kinase
pVHL: von Hippel-Lindau protein
RAS: rat sarcoma
TCA: tricarboxylic acid
TMA: tissue micro array
TTF-1: thyroid transcription factor 1
VEGF: vascular endothelial growth factor
WT1: Wilms tumor protein 1

1. Abstract

Background: Malignant pleural mesothelioma (MPM) is a therapy refractory tumor of the pleural lining mesothelial cells. As of today not much is known about the molecular pathology of MPM. The hypoxia inducible factors (Hif) have been implicated in the pathogenesis of many solid tumors. Its stabilization leads to a glycolytic phenotype and angiogenesis, two hallmarks of cancer. This study aimed to identify a correlation between expression of Hif1α and one of its target genes glucose transporter 1 (Glut1) with survival of MPM patients.

Patients and Methods: Between 1999 and 2009 a prospective patient cohort containing 192 patients diagnosed with MPM was investigated on tissue micro arrays (TMA) by immunohistochemistry. Four TMAs were constructed, two with pre-chemotherapy biopsies and another two with corresponding tissue from the post-chemotherapy extrapleural pleuro-pneumonectomy (EPP) or decortication and debulking specimens, respectively. Nuclear Hif1α and membranous Glut1 immunoreactivity was scored semi-quantitatively. The patients were divided into two groups according to there average score, one group with no/very low protein levels and one group with medium/high protein expression. These two groups were then statistically analyzed for overall survival, tumor stage and histology.

Results: 140 patients could be analyzed from the pre- and 141 from the post-chemotherapy TMAs. In the pre-chemotherapy samples the survival time was correlated to Hif1α expression. Comparing any Hif1α expression to no expression, median survival was significantly shorter (log rank test *p=0.025*). This association could not be found in the post-chemotherapy samples (p=0.59). For the Glut1 expression there was a trend visible towards shorter survival of the patients with higher protein levels, however there was no statistical significance (log rank test pre-chemotherapy samples p=0.19 and for post-chemotherapy samples p=0.06). The more advanced tumor stages showed higher expression of Glut1 (Chi-square test p=*0.027* for T stage and p=*0.012* for IMIG stage respectively). A similar correlation could not be observed for Hif1α.

Conclusion: This thesis adds to the evidence that the expression of Hif1α and the glycolytic phenotype could play an important role in the progression of MPM. Concerning survival, high expression of Hifα and Glut1 may be associated with shorter survival, although significance of these results may be influenced by the neo-adjuvant chemotherapy preceeding surgery.

2. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive and therapy resistant tumor of the mesothelial cells lining the pleural cavity, which increases in frequency throughout the world. In the canton of Zurich 30 new cases are diagnosed every year with an incidence of 3.7/100000 in men and 0.6/100000 in women (Ceschi et al., 2009). With a 5-year survival rate of 10,3% the prognosis is still very poor (diagnosis 1995-99). The majority of the patients have a history of occupational asbestos exposure (Ceschi et al., 2009). Patients with mesotheliomas typically present with pleural effusion, which is often associated with chest wall pain. B-symptoms like fatigue or weight loss can also be present. Occasionally, patients have no symptoms and the disease is diagnosed on a routine chest radiograph (Robinson et al., 2005).
There are three different histologic subtypes of mesotheliomas: Epithelioid mesothelioma resembling tissue of epithelial origin, sarcomatoid mesothelioma with a spindle cell character and biphasic mesothelioma with parts of both types (Figure 3). The epithelioid type accounts for approximately 60%, sarcomatoid for 20% and biphasic ranges around 20% of all mesotheliomas (Sekido, 2010). The morphological diagnosis of pleural mesothelioma is generally made by a pleural biopsy, preferentially by thoracoscopy. To distinguish between lung adenocarcinoma and epithelioid mesothelioma immunohistochemistry is important. Markers for an epithelioid mesothelioma are calretinin, D2-40, Wilms tumor protein 1 (WT1), cytokeratin 5/6 and podoplanin, while carcino-embryonic antigen (CEA), epithelial membrane antigen (EMA) and thyroid transcription factor 1 (TTF-1) are markers for the diagnosis of adenocarcinoma and are almost never expressed in malignant mesothelioma. As of today there is no standard therapy for patients with malignant pleural mesothelioma. However a multicenter study conducted in Switzerland including neoadjuvant chemotherapy with cisplatin/gemcitabine or cisplatin/pemetrexed followed by extrapleural pleuro-pneumonectomy (EPP) showed good results compared to historical outcomes (Weder, 2010).
The molecular pathology of malignant pleural mesothelioma has an unusual pattern with predominant loss of tumor suppressor genes, especially the $p14^{INK4A}$, $p16^{ARF}$, and neurofibromin 2 (NF2) genes. Well-known oncogenes like rat-sarcoma (RAS) or phosphoinositide-3-kinase (PI3K) are not frequently mutated. However activation of the upstream receptor tyrosine kinases including the family of epidermal growth factor receptors (EGFR) or hepatocyte growth factor receptor (HGFR) with subsequent activation of mitogen-activated protein (MAP) kinase and PI3K signaling cascades are

frequently identified in mesothelioma cells (Sekido, 2010). For an overview of oncogenic signaling through receptor tyrosine kinases see Figure 1.

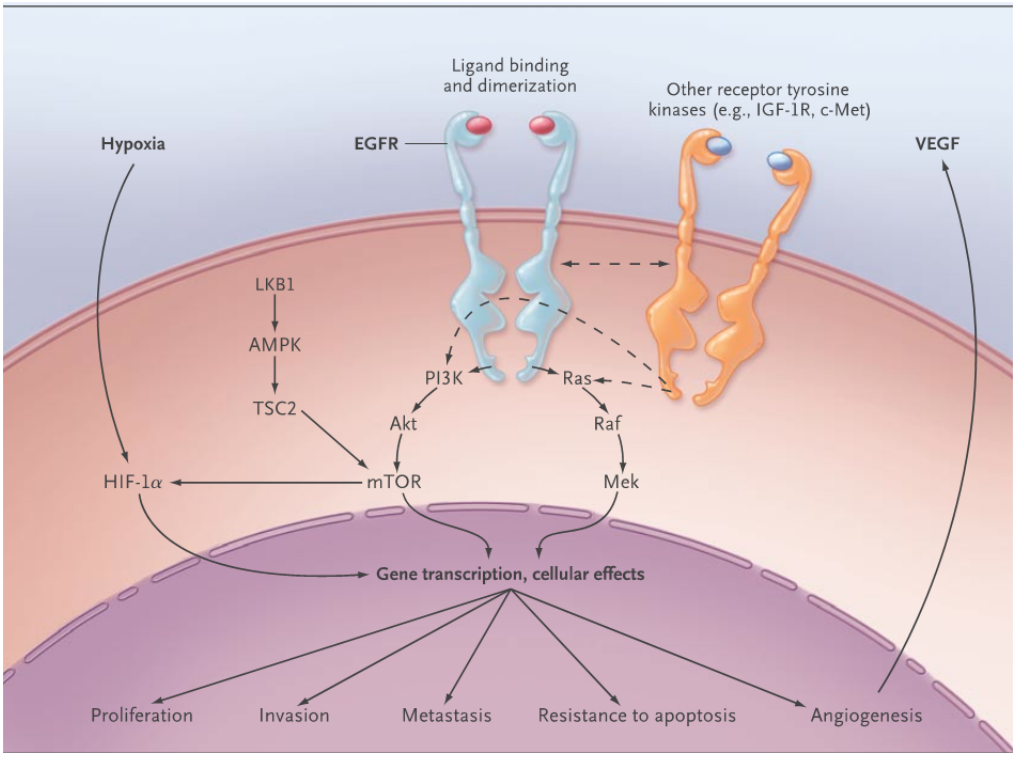


Figure 1: (Herbst et al., 2008) Oncogenic signaling through the EGFR receptor: In normal cells EGFR is activated through binding of a growth factor ligand. This binding leads to a dimerization of EGFR and thereby to an activation of the downstream signaling pathways like the MAP-Kinase or the PI3-Kinase pathway, which leads to cell survival and proliferation. In cancer cells a point mutation in a gene encoding one of this signaling proteins can lead to a constitutive activation of the signaling cascade. This leads to uncontrolled proliferation of the cell and can contribute to other hallmarks of cancer like angiogenesis, invasion and metastasis.

Hypoxia inducible factor 1α (Hif1α) is a transcription factor that is activated upon low tissue oxygenation. In normoxia Hif1α is hydroxylated on proline residue 402 and/or 564 by prolyl hydroxylases (PHD). This modification leads to the binding of the von Hippel-Lindau (pVHL) E3 ligase complex and to the subsequent ubiquitination and proteasomal degradation of Hif1α (Wang et al., 1995). During hypoxia stable Hif1α heterodimerizes with Hif1β (also known as aryl hydrocarbon receptor nuclear translocator protein, ARNT) and is imported into the nucleus, where it leads to the transcription of glycolytic genes

(pyruvate dehydrogenase kinase (PDK), lactate dehydrogenase (LDH) and glucose transporter 1 (Glut1) as well as to the induction of angiogenesis via expression of vascular endothelial growth factor (VEGF) (Semenza, 2010). During cancer progression rapid proliferation of cancer cells often outgrow blood supply. This leads to low oxygen concentration in the tumor tissue and to an up-regulation of Hif1α. Immunohistochemical analysis of human cancer biopsies showed increased levels of Hif1α protein in the majority of primary human cancers and their metastases (Zhong et al., 1999). It has been shown that increased levels of Hif1α expression, determined by immunohistochemistry not only is associated with increased patient mortality in a variety of human cancers but also correlates with chemo- and radiotherapy resistance (see Semenza 2010 for an overview of the literature). Loss of function of many tumor suppressors leads to a stabilization of the Hif1α protein. The most famous example is loss of VHL protein in clear cell renal cell cancer. This leads to constantly activated Hif1α and thereby to increased angiogenesis and a glycolytic phenotype (Maxwell et al., 1999). It was demonstrated that other factors apart from oxygen like iron (Fe), ascorbate, and various tricarboxylic acid (TCA) cycle intermediates can affect proline hydroxylase activity (Pan et al., 2007) (see Figure 2) Activation of oncogenic signaling through the PI3K/AKT or MAP-Kinase pathway also increases Hif1α synthesis through the activation of mammalian target of Rapamycin (mTOR) (Laughner et al., 2001).

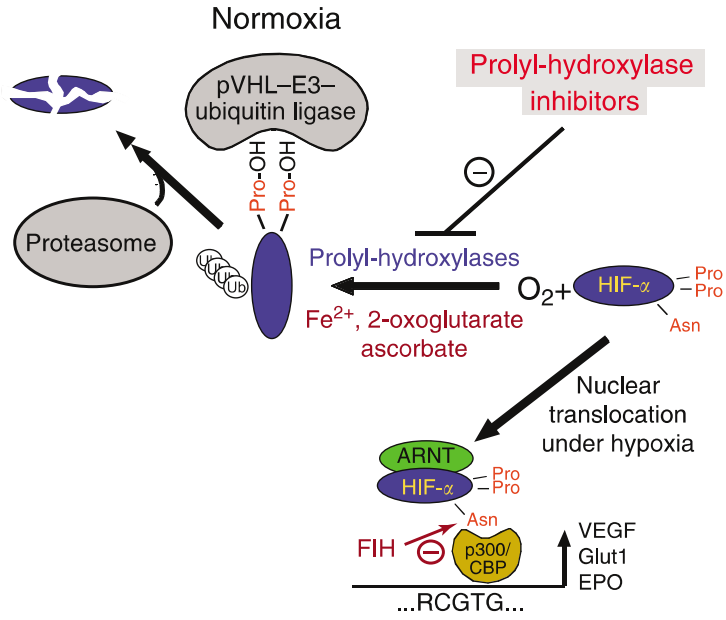


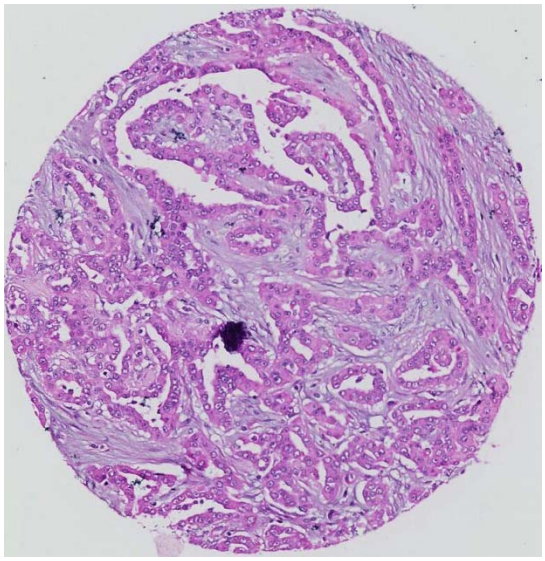
Figure 2: (Haase, 2006) Hif1α is a very unstable protein. Under normoxic conditions it is constantly ubiquitinated via the pVHL-E3-ubiquitin ligase complex and degraded by the proteasome. Under low oxygen concentrations Hif1α is stabilized and can translocate to the nucleus, where it leads to the transcription of its target genes. For pVHL binding Hif1α has to be hydroxylated on proline residues. For this reaction cofactors like reduced iron (Fe^{2+}) and the TCA intermediate α-ketoglutarate are necessary.

Glucose transporter 1 (Glut1) is one of the most important target genes of Hif1α. Glut1 is expressed in almost all human tissues and takes up glucose into the cell in an Insulin-independent way. Already in 1930 Otto Warburg described an up-regulated glycolytic activity in cancer (Warburg, 1956). This phenomenon of elevated glycolysis even in the presence of oxygen is known as the 'Warburg effect'. Nowadays the elevated glucose consumption rate of cancer is used in ^{18}FDG-PET for diagnostic purposes and can be seen as a hallmark of cancer (Hanahan and Weinberg, 2011). The fact that the glycolytic phenotype in primary and metastatic cancers persists also in the presence of oxygen indicates that it confers a selective growth advantage to the cancer cell. It has been shown that oncogenic up-regulation of the PI3K/AKT and mTOR/S6K pathway leads to increased glycolysis and glucose consumption (reviewed in Gatenby and Gillies, 2004).
Gene expression profiling of mesothelioma tissue specimens from 16 patients and 4 control samples by cDNA microarray showed the most significant up-

regulation in glycolytic genes. Lactate dehydrogenase (LDH) was up-regulated 5-fold and there was a strong and significant positive correlation with Hif1α levels (r^2=0.98, Spearman coefficient, P < 0.05). This suggests a role for Hif1α in the pathogenesis of malignant pleural mesothelioma (Singhal et al., 2003). It was shown that a 24h incubation of mesothelioma cells with crocidolite asbestos fibres leads to an activation of Hif1α and to a reduced sensitivity to doxorubicin treatment. It was proposed that crocidolite leads to an iron chelation at the fibre surface and decreases iron availability in the cells, a situation that would subsequently give rise to an activation of Hif1α (Riganti et al., 2008). In a small study with 34 specimens from patients with malignant pleural mesothelioma it was shown that Hif1α is commonly expressed in the tumor tissue but not in normal mesothelium. However the authors could not show a statistically significant effect of Hif1α expression on prognosis of patient survival (Klabatsa et al., 2006).

There is a lot of evidence for a role of Hif1α levels and the emerging glycolytic phenotype in the pathogenesis of malignant pleural mesothelioma. Until now there is no study published on a large patient collective about the prognostic significance of Hif1α and Glut1 protein expression levels. For this purpose a tissue microarray with tissue specimens from approximately 200 patients with pre- and post-chemotherapy samples was constructed.

The aim of this thesis is to study the correlation of Hif1α as well as Glut1 protein expression levels with patient survival, using the tissue microarray technology.



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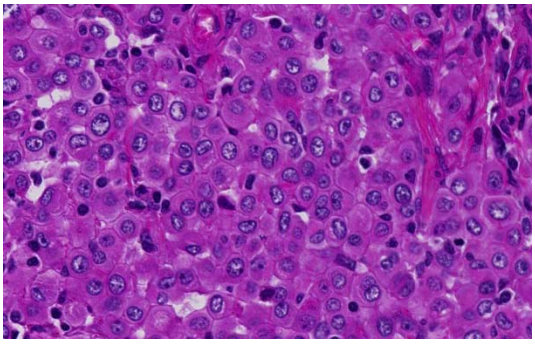

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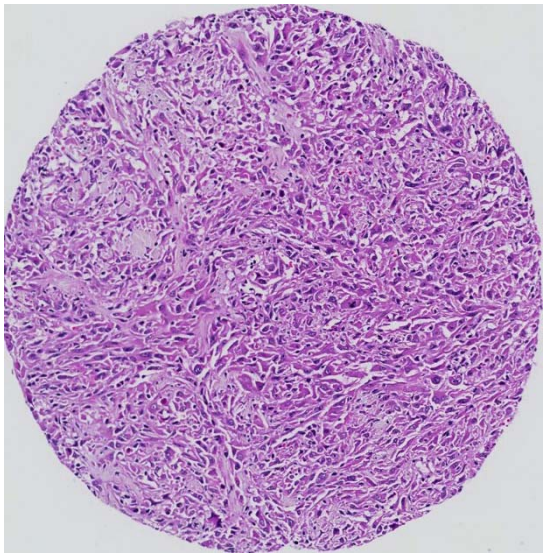

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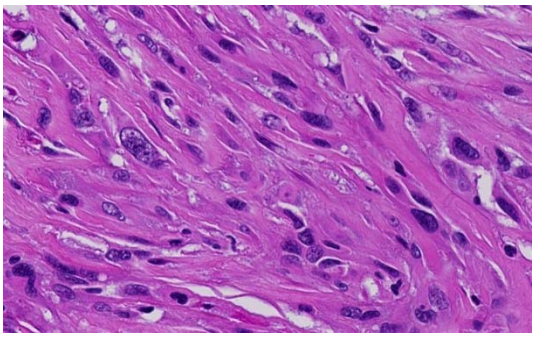

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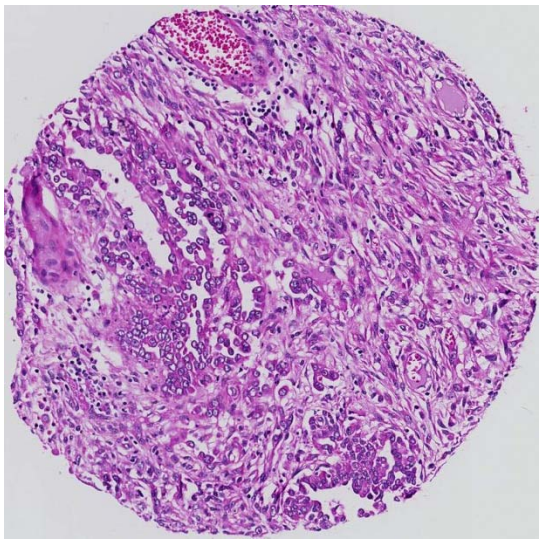

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Figure 3: Histologic subtypes epithelioid mesothelioma A 10x; B 40x; sarcomatoid mesothelioma C 10x, D 40x; biphasic mesothelioma E 10x

3. Patients and methods

3.1 Patients and tissue collection

For the time period of 1999 to 2009 a total of 192 patients with malignant pleural mesothelioma diagnosed by thoracoscopy were investigated. Once diagnosed the patients were assigned to the University Hospital of Zurich, were they received neoadjuvant chemotherapy and most of them (147) underwent extrapleural pleuro-pneumonectomy, decortication or debulking thereafter. For the pre-chemotherapy analysis 52 of the patients were excluded because of lack of material. From the 147 patients that underwent post-chemotherapy surgery 6 were excluded because of insufficient material. Additional information such as age, gender and staging were gathered from pathology reports. Pathological staging was done as recommended by the IMIG (International Mesothelioma Interest Group) staging system (see Table 2) (Wittekind et al., 2010). Staging was available from 153 patients. Survival and follow-up data was gathered from the reports of the Thoracic Surgery department. The average patient age was 61 and ranged form 35 to 93. Twenty patients were female and 172 male. An overview of the pathological and clinical parameters as well as hospitals of first diagnosis can be found in Table 1.

Table 1: Pathological and clinical parameters

		tissue sample	
		n	%
Total		192	100
Hospital diagnosed	Universitätsspital Zürich	121	63
	Stadtspital Triemli	8	4
	Kantonsspital Luzern	6	3
	Kantonsspital Winterthur	15	8
	Kantonsspital Aarau	6	3
	Kantonsspital Baden	7	4
	Kantonsspital Graubünden	2	1
	Kantonsspital St. Gallen	5	3
	Universitätsspital Basel	3	2
	Inselspital Bern	4	2
	Kantonsspital Münsterlingen	11	6
	others	4	2
Histology	epithelioid	102	53
	sarkomatoid	8	4
	biphasic	51	27
Tumor stage	pT1	11	8
	pT2	53	40
	pT3	56	43
	pT4	11	8
Nodal status	pNx	1	1
	pN0	83	63
	pN1	18	14
	pN2	29	22
	pN3	0	0
Metastases	pMx	66	50
	pM0	64	49
	pM1	1	1
IMIG stage	I	11	7
	II	31	20
	III	79	52
	IV	32	21

Table 2: IMIG staging system for malignant pleural mesothelioma

Primary tumor (T):

- **T1:** Tumor involves pleura parietalis of same side (T1b + focal involvement of pleura visceralis).
- **T2:** Tumor involves same side pleura with at least one of the following features: Confluent tumor on pleura visceralis, involvement of the muscles of the diaphragm or the lung tissue deeper to the mesothelium covering the lung.
- **T3:** Involvement of the endothoracic fascia, the mediastinal fat or single focus of tumor involving the soft tissue of the chest wall
- **T4:** Invasion of any mediastinal organ, diffuse or multi-focal involvement of the soft tissue of the chest wall, rib, peritoneum, pleura of the other site, malignant pericardial effusion or involvement of heart muscle

Lymph node involvement (N):

- **N0:** No regional lymph node involvement
- **N1:** Involvement of same side bronho-pulmonary and or hilar lymph nodes only
- **N2:** Involvement of subcarinal lymph node(s), and or same side or opposite side internal mammary or mediastinal lymph node(s)
- **N3:** Involvement of opposite side mediastinal, internal mammary, or hilar lymph node(s) and or same side or opposite side supraclavicular or scalene lymph node(s)

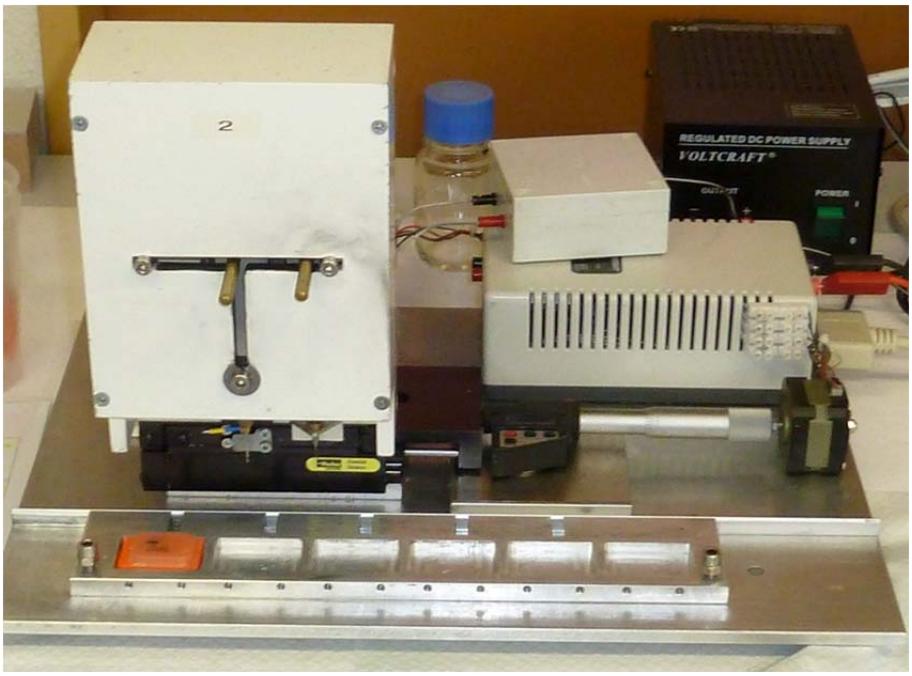
Distant metastasis (M):

- **Mx:** Distant metastasis cannot be assessed
- **M0:** No distant metastasis
- **M1:** Distant metastasis present

IMIG-Stage:
Stage I: T1 N0 M0 (Stage IA: T1a N0 M0, Stage IB: T1b N0 M0)
Stage II: T2 N0 M0
Stage III: T1, T2 N1 M0 or T1, T2 N2 M0 or T3 N0, N1, N2, M0
Stage IV: T4 any N M0 or any T N3 M0 or any T any N, M1

3.2 Construction of TMA

Four tissue microarrays (TMA) were constructed according to an established method (Kononen et al., 1998). Hematoxylin-Eosin stained sections of thoracoscopic biopsies and surgical speciments were reviewed and representative areas were selected. Tissue cylinders with a diameter of 0.6 mm were punched out from those areas of the corresponding paraffin blocks. We constructed 2 TMAs from the thoracoscopic biopsies with 2 tissue cylinders per patient as well as 2 TMAs from surgical specimens with 4 tissue cylinders per patient sample. Additionally, on the 2 surgical specimen TMAs 20 punches of different control tissues, including normal mesothelium, adenocarcinoma of the lung, lymphoid tissue and 4 different mesothelioma cell lines were collected. With the help of an automatic tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA) the TMAs were constructed by precisely arraying the tissue cylinders in a new paraffin block. Figure 4 shows the device and an example of a TMA.



A

B

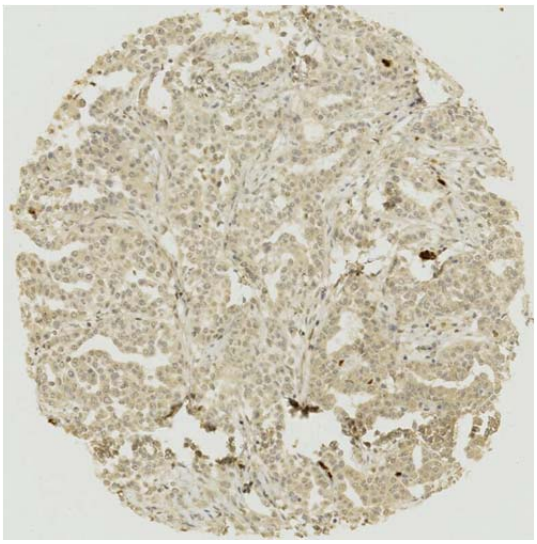
Figure 4: A: Tissue array device for the construction of the TMA. B: Overview of the tissue microarray block.

3.3 Immunohistochemistry

For immunohistochemical analysis, 4-μm-thick paraffin sections were cut from the TMA block and mounted on silane coated glass slides. After incubation at 37°C overnight, deparaffinization in xylene and rehydration through graded ethanol series were performed. For the Glut1 staining the rabbit polyclonal antibody AB1341 (Chemicon/Millipore Corporation) was used at a 1:1000 dilution on a Ventana Benchmark® platform (Ventana Medical Systems, Tucson, AZ, USA), the cell conditioner 1 (CC1) standard mono protocol (CC1-mono) was performed: pre-treatment with boiling for 60 min in pH 8 Tris buffer, incubation with primary ab for 60 min at room temperature (RT) and development with the Ultraview- HRP mono kit, including incubation with respective secondary ab for 30 min at RT. For Hif1α a mouse monoclonal antibody (Abcam, Cambridge UK) was used in a 1:400 dilution on a Leica Bond® platform (Vision Biosystems, Melbourne, Australia), the H2 standard (H2-60) protocol was performed: pre-treatment with boiling for 60 min in pH 8 Tris buffer, incubation with primary ab for 30 min at RT.

3.4 Interpretation of results

For each core the intensity of immunoreactivity was scored semiquantitatively. For Hif1α nuclear staining was scored from 0 to 3 (0=no staining, 1=weak staining, 2=moderate staining, 3=strong staining). Glut1 scoring was performed for intensity and frequency. Intensity was scored the same way like for Hif1α, frequency was scored as a percentage of stained cancer cells (0%=0, 1-9%=0.1, 10-49%=0.5, 50-100%=1). Out of this two scores an H-score was calculated as intensity multiplied with frequency. Figure 5 shows examples of weak and strong staining of Hif1α and Glut1 samples.



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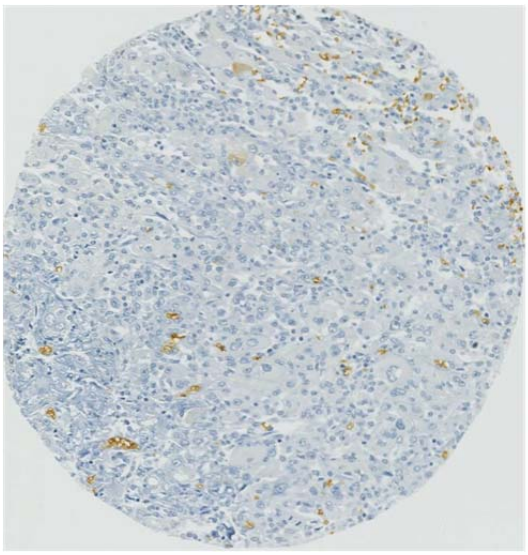

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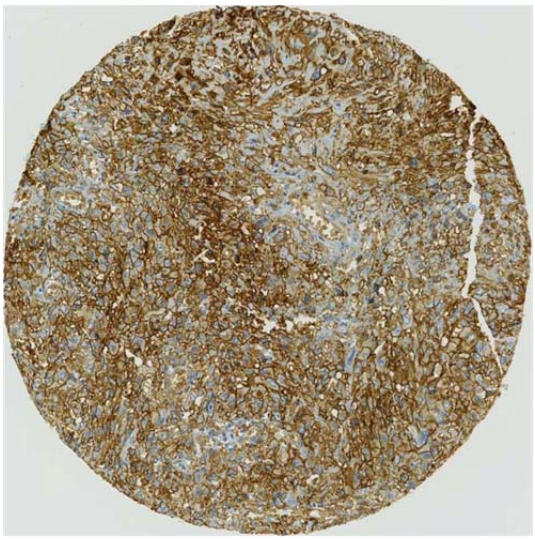

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Figure 5: Hif1α staining: A: no; B: strong; Glut1 staining: C: no; D: strong 10x

3.4 Statistical analysis

The correlation between clinicopathological findings and immunohistochemical parameters was calculated by Chi-squared tests.
For all investigations with respect to patient prognosis, the overall survival was chosen as the endpoint. For calculation of the survival time, living patients were evaluated as "censored".
The illustration of the cumulative survival curves was performed by the method of *Kaplan-Meier*. A *log rank* test was used for the statistical assessment of differences between cumulative survival curves. Statistical analyses were performed using the statistical software package *SPSS-statistics 19* (*SPSS Inc., Chicago, IL, USA*). *P*-values < 0.05 were considered statistically significant in all analyses.

4. Results

4.1 Overall and median survival

There was a significant association between IMIG-Stage and overall survival rates (*p=0.013*). Figure 6 shows the survival curves for the IMIG-Stages.
The median survival rates were very poor with an estimate of 22 month for all patients and respective median survival rates of 40 month in Stage I, 26 month in Stage II, 21.5 month in Stage III and 11.5 month in Stage IV patients.

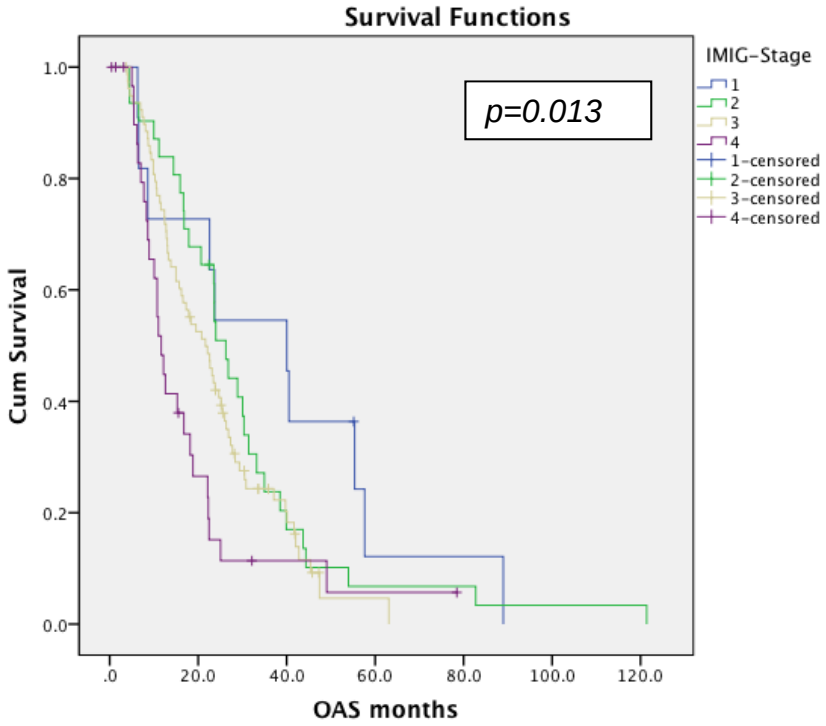

Figure 6: IMIG-Stage and Overall survival

4.2 Immunohistochemical findings

In order to evaluate the correlation of protein expression and survival rates the results obtained from TMA scoring for Hif1α and Glut1 were divided into two equal groups (no/low expression and moderate/strong) and compared with the survival rates of the patients. The pre- and post-chemotherapy results were analysed independently.

4.2.1 Hif1α

4.2.1.1 Pre-chemotherapy samples

From the 140 analysed patients from the pre-chemotherapy samples 83 had no immunohistochemical detectable Hif1α protein. The survival of these patients was compared to the 57 with Hif1α expression. Overall survival of the patients with no Hif1α expression was significantly longer than that of the patients with Hif1α expression (*p=0.025*). There was no association with tumor histology detectable.

Table 3: Mean Hif1α score in pre-chemotherapy samples

Hif1α mean score	Total N	N of Events	Censored	
			N	Percent
= 0	83	70	13	15.7%
1-3	57	48	9	15.8%
Overall	140	118	22	15.7%

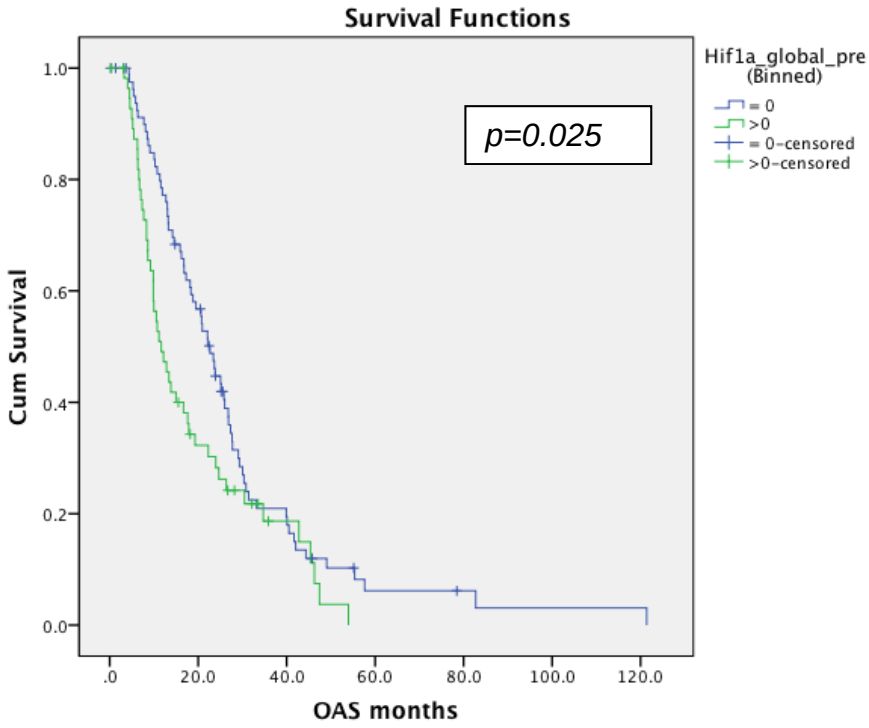

Figure 7: Hif1α expression in pre-chemotherapy samples and overall survival

4.2.1.2 Post-chemotherapy samples

In the post chemotherapy samples from the 141 analysed, 82 had an average staining of less than 0.5 and 59 patients had a staining of more than 0.5. There was no statistically significant difference in overall survival between these two groups (p=0.59). There was a statistically significant correlation towards less Hif1α expression in sarcomatoid and biphasic mesothelioma as well as higher tumor stages. For IMIG stage there was no statistically significance but also a trend to less Hif1α expression in advanced stages (Table 5).

Table 4: Mean Hif1α score in post-chemotherapy samples

Hif1α mean score	Total N	N of Events	Censored	
			N	Percent
</= 0.5	82	72	10	12.2%
>0.5	59	55	4	6.8%
Overall	141	127	14	9.9%

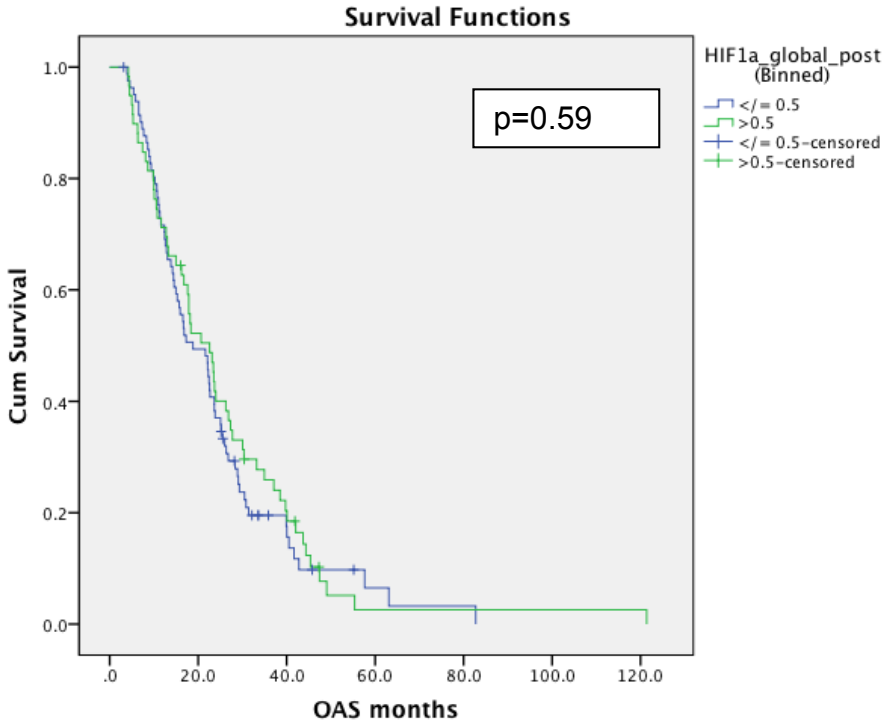

Figure 8: Hif1α expression in post-chemotherapy samples and overall survival

Table 5: Hif1α expression and histopathological parameters.

			Hif1α expression	
			≤0.5	>0.5
		n	n(%)	n(%)
Histology	epithelioid	77	42(55%)	35 (45%)
	sarcomatoid	8	5(62.5%)	3(37.5%)
p=0.046	biphasic	56	35(62.5%)	21(37.5%)
Tumor stage	pT1	11	6(55%)	5(45%)
	pT2	44	21(48%)	23(52%)
	pT3	53	35(66%)	18(34%)
p=0.042	pT4	20	12(60%)	8(40%)
IMIG stage	1	10	6(60%)	4(40%)
	2	29	13(45%)	16(55%)
	3	71	45(63%)	26(37%)
p=0.054	4	21	13(62%)	8(38%)

4.2.2 Glut1

4.2.2.1 Pre-chemotherapy samples

The 140 patients analysed for Glut1 expression were divided into two almost equal groups. 75 patient samples showed no or almost no immunohistochemicaly detectable expression of Glut1 (mean H score ≤0.3) and 65 patients showed some expression (mean H score >0.3). Between this two groups there was no statistically significant difference in overall survival (p=0.19). There was no association with tumor histology detectable.

Table 6: Mean Glut1 H-score in pre-chemotherapy samples

Glut1 mean H-score	Total N	N of Events	Censored	
			N	Percent
</= 0.3	75	63	12	16.0%
>0.3	65	55	10	15.4%
Overall	140	118	22	15.7%

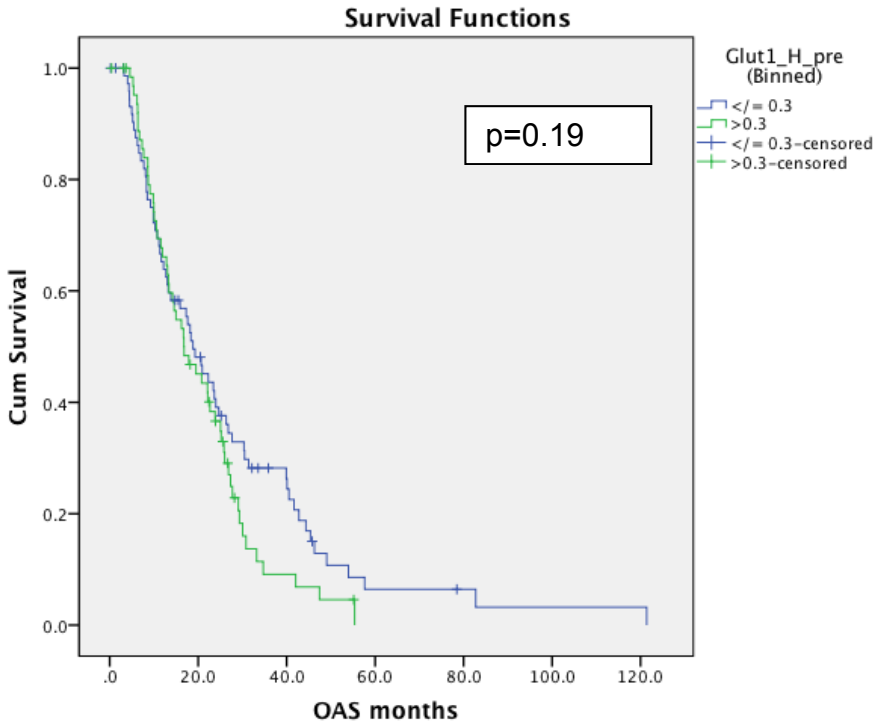

Figure 9: Glut1 expression in pre-chemotherapy samples and overall survival

4.2.2.2 Post-chemotherapy samples

From the 141 patient samples analysed 78 had no detectable Glut1 staining and 63 had some Glut1 staining detectable (Table 7). There was no statistically significant difference in overall survival detectable between these two groups (p=0.06). But a Chi-square test showed a statistically significant correlation between Glut1 expression and tumor as well as IMIG stage with p-values of *0.027* and *0.012* respectively (Table 8). No differences in Glut1 expression between the histological subtypes could be detected. There was no correlation between Glut1 and Hif1α expression with a Pearson-coefficient of -0.123 (p=0.145).

Table 7: Mean Glut1 H-score in pre-chemotherapy samples

Glut1 mean H-score	Total N	N of Events	Censored	
			N	Percent
= 0	78	71	7	9.0%
1-3	63	56	7	11.1%
Overall	141	127	14	9.9%

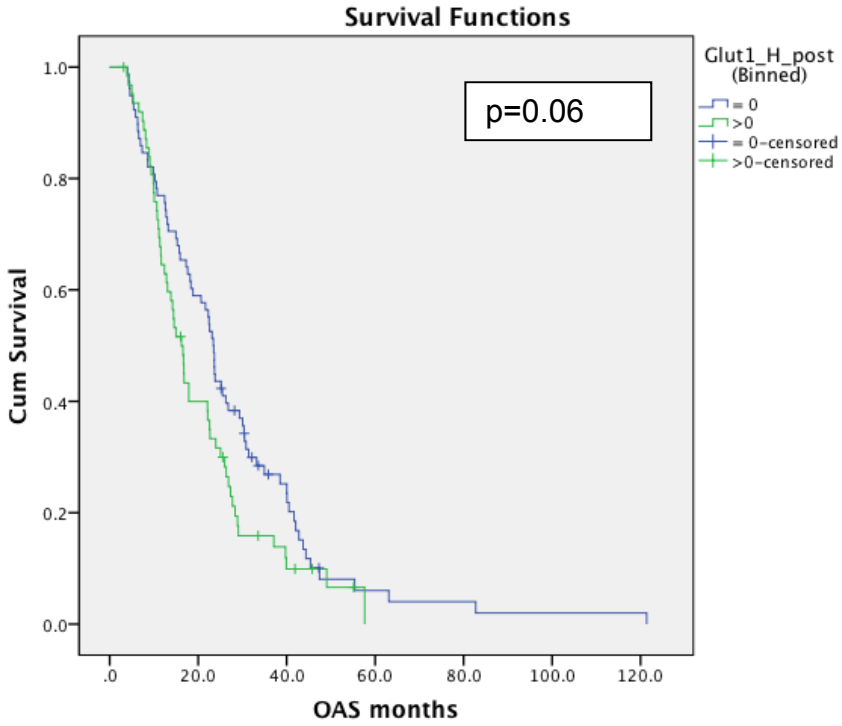

Figure 10: Glut1 expression in post-chemotherapy samples and overall survival

Table 8: Glut1 expression and histopathological parameters.

			Glut1 expression	
			=0	1-3
		n	n(%)	n(%)
Histology	epithelioid	77	44(57%)	33 (43%)
	sarcomatoid	8	5(62.5%)	3(37.5%)
p=0.059	biphasic	56	29(52%)	27(48%)
Tumor stage	pT1	11	7(63.5%)	4(36.5%)
	pT2	44	27(61%)	17(39%)
	pT3	53	22(41.5%)	31(58.5%)
p=0.027	pT4	20	10(50%)	10(50%)
IMIG stage	1	10	8(80%)	2(20%)
	2	29	19(65.5%)	10(34.5%)
	3	71	37(52%)	34(48%)
p=0.012	4	21	10(47.5%)	11(52.5%)

5. Discussion

5.1 Hif1α expression and glycolytic phenotype in human cancers

It has been shown that Hif1α is overexpressed in many human cancers (Zhong et al., 1999) and linked to higher mortality in a wide variety of human cancers (Aebersold et al., 2001; Bachtiary et al., 2003; Birner et al., 2000; Burri et al., 2003; Dales et al., 2005; Generali et al., 2006; Giatromanolaki et al., 2004; Giatromanolaki et al., 2001; Giatromanolaki et al., 2003; Korkolopoulou et al., 2004; Kronblad et al., 2006; Rajaganeshan et al., 2008; Rasheed et al., 2009; Sivridis et al., 2002; Theodoropoulos et al., 2005; Theodoropoulos et al., 2004; Trastour et al., 2007; Tzao et al., 2008; Vleugel et al., 2005; Yamamoto et al., 2008). The transcription factor Hif1α leads to the expression of genes involved in glycolysis and angiogenesis, both of which can be seen as hallmarks of cancer (Gatenby and Gillies, 2004). There is some weak evidence that overexpression of Hif1α and glycolytic genes like Glut1 could also play a role in the pathogenesis of MPM and that the Hif1α stabilization in mesothelioma cells could be linked to asbestos fibres that are deposited in the pleural lining (Riganti et al., 2008; Sekido, 2010; Singhal et al., 2003). However in a small study on biopsy samples from 45 patients with a malignant pleural mesothelioma no prognostic significance of Hif1α on patient survival could be observed (Klabatsa et al., 2006).

5.2 TMAs for analysis of molecular tumor markers

To analyze a larger patient cohort we used a tissue microarray (TMA) based approach and constructed 4 TMAs with samples of pre- and post-chemotherapy specimens of 192 patients. A TMA allows an efficient analysis of molecular markers in human malignant tumors. So that it is possible to compare the immunohistochemical staining for a protein on a large number of patient samples on one slide. This does not only have the advantage that it safes costs but also the conditions for the immunohistochemical staining is identical for all tissue samples. A limitation of this technique is that the small core diameter might not be representative for an entire tumor (Shergill et al., 2004). However excellent correlations have been reported between data obtained from TMA compared to whole section immunostaining in a wide

range of human tumors (Hoos et al., 2001; Mucci et al., 2000; Nocito et al., 2001; Rubin et al., 2002; Zhang et al., 2003).
To limit the possible effect of different protein expression in different regions of the tumor we used 2 punch cores in the pre-chemotherapy biopsies and 4 in the pleuro-pneumonectomy specimens.
To get a patient sample that is large enough for a rare disease like MPM we included all patients operated at the University hospital of Zurich since 1999. Many of them got their diagnosis at another hospital somewhere else in Switzerland or even in Italy (see Table 1). The large distribution of the hospitals where the patients got their diagnosis, made it difficult to obtain the complete clinical information and the tissue samples needed to construct the TMAs. This may also explain the high number of patients excluded from either the pre- or post-chemotherapy analysis.

5.3 Higher Hif1α expression is associated with poorer patient survival in the pre-chemotherapy samples and higher Glut1 expression with advanced tumor stages

The results of our study showed a significant effect towards poorer survival of the patients with detectable Hif1α levels, but only in the pre-chemotherapy samples. Although a trend towards dismal survival of patients with Hif1α expression was also visible in the post-chemotherapy samples (Figure 8) it was not statistically significant. Interestingly there was a trend to lower Hif1α protein levels in more advanced stages. Albeit this was only statistically significant for T stages, this finding is very surprising. It may be explained by general progressive genomic instability of MPM during tumor enlargement. Further, histotype and chemotherapy may be relevant influencing factors.
To further investigate the role of Hif1α and the consequent glycolytic phenotype in MPM we stained the 4 TMAs for one of the most important Hif-target genes: Glucose transporter 1. Neither for the pre- nor the post-chemotherapy samples we could find a statistically significant correlation between patient survival and Glut1 expression. A weak trend towards poorer survival of patients with higher Glut1 expression however is visible (Figure 9,10). We could also observe a statistically significant correlation towards higher Glut1 expression in more advanced stages (Table 8). In our patient sample there was no correlation between Hif1α and Glut1 expression. This surprising finding could be explained by a possible overexpression of the second Hifα isoform Hif2α. A Hif2α staining of the TMAs should be performed

in the future to check for a possible correlation between Glut1 and Hif2α levels. Another explanation could be that the Glut1 expression in MPM is more linked to oncogenic signaling via the MAP-Kinase or PI3K mTOR pathways.

One thing that has always to been taken into consideration when working with Hif1α is, that it is a very unstable protein and its levels could be affected by differences in handling of the tissue. Already during the operation different times of malperfusion could affect Hif1α protein levels and consequently the levels of its target genes.

In general, the statistically significant effects towards higher Glut1 expression in more advanced stages as well as the poorer survival of patients with Hif1α protein expression observed add to the evidence that the glycolytic phenotype could play a role in the pathogenesis of MPM. In the future it will be very important to combine TMA based studies with cell culture experiments and exome sequencing of primary tumors. Thereby, it may be possible to further elucidate the molecular mechanisms contributing to the changes observed in the metabolism of MPM cells. This could possibly lead to the discovery of novel therapeutic targets for patients with MPM (Michelakis et al., 2008).

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